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# Antifungal Susceptibility of *Candida* Biofilms: Unique Efficacy of Amphotericin B Lipid Formulations and Echinocandins

D. M. Kuhn,[1,2] T. George,[2] J. Chandra,[2] P. K. Mukherjee,[2] and M. A. Ghannoum[2]*

*Division of Infectious Diseases, Department of Medicine,[1] and Center for Medical Mycology, Department of Dermatology,[2] University Hospitals of Cleveland and Case Western Reserve University, Cleveland, Ohio 44106*

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**Biofilms, likely the predominant mode of device-related microbial infection, exhibit resistance to antimicrobial agents. Evidence suggests that *Candida* biofilms have dramatically reduced susceptibility to antifungal drugs. We examined antifungal susceptibilities of *Candida albicans* and *Candida parapsilosis* biofilms grown on a bioprosthetic model. In addition to conventional agents, we determined if new antifungal agents (triazoles, amphotericin B lipid formulations, and echinocandins) have activities against *Candida* biofilms. We also explored effects of preincubation of *C. albicans* cells with subinhibitory concentrations (sub-MICs) of drugs to see if they could modify subsequent biofilm formation. Finally, we used confocal scanning laser microscopy (CSLM) to image planktonic- and biofilm-exposed blastospores to examine drug effects on cell structure. *Candida* biofilms were formed on silicone elastomer and quantified by tetrazolium and dry weight (DW) assays. Susceptibility testing of fluconazole, nystatin, chlorhexidine, terbenafine, amphotericin B (AMB), and the triazoles voriconazole (VRC) and ravuconazole revealed resistance in all *Candida* isolates examined when grown as biofilms, compared to planktonic forms. In contrast, lipid formulations of AMB (liposomal AMB and AMB lipid complex [ABLC]) and echinocandins (caspofungin [Casp] and micafungin) showed activity against *Candida* biofilms. Preincubation of *C. albicans* cells with sub-MIC levels of antifungals decreased the ability of cells to subsequently form biofilm (measured by DW; $P < 0.0005$). CSLM analysis of planktonic and biofilm-associated blastospores showed treatment with VRC, Casp, and ABLC resulted in morphological alterations, which differed with each agent. In conclusion, our data show that *Candida* biofilms show unique susceptibilities to echinocandins and AMB lipid formulations.**

Biofilms represent the most prevalent type of microbial growth in nature and are crucial to the development of clinical infections (18, 38). They can serve as a nidus for disease and are often associated with high-level antimicrobial resistance of the associated organisms (32). *Candida* is the fourth most common cause of bloodstream infection in hospitalized patients (8). Up to 40% of patients with *Candida* isolated from intravenous catheters have underlying fungemia (2, 36), and the mortality rate of patients with catheter-related candidemia approaches 40% (36). While *Candida albicans* is the most commonly isolated fungal species, others species are being isolated with increasing frequency (27, 41). *Candida parapsilosis* has become the second most commonly isolated fungal organism in several studies (26, 39). This species is of particular concern in critically ill neonates, where it is known to be associated with central lines and parenteral nutrition (48, 49, 57).

Candidiasis associated with intravenous lines and bioprosthetic devices is especially problematic, since these devices can act as substrates for biofilm growth. Antifungal therapy alone is insufficient for cure; affected devices generally need to be removed (34, 36, 37, 47). Removal of these devices has serious implications in the case of infected heart valves, joint prostheses, and central nervous system shunts. The reason for the need for device removal has been, until recently, a mystery. However, our lab and others have demonstrated a near-total resistance to antifungal agents by biofilm-associated *Candida* (6, 13, 14, 21, 29).

In this study, we set out to answer a number of fundamental questions about bioprosthesis-related *Candida* biofilms and their profound drug resistance. First, we undertook a comprehensive examination of the antifungal susceptibility of *C. albicans* biofilms grown on our bioprosthetic model (29). Second, we extended these susceptibility studies to *C. parapsilosis*, given this organism's prevalence in neonatal line-related *Candida* infections. Third, we determined if new antifungal agents, including the triazoles, lipid-formulated amphotericin B, and echinocandins, have any unique activities against *Candida* biofilms. Fourth, we explored the effects of preexposure to subinhibitory concentrations (sub-MICs) of antifungal drugs to determine if pretreatment with these drugs could modify subsequent biofilm formation and metabolism. Finally, we used confocal scanning laser microscopy (CSLM) to image the effects, on cell structure, of drug exposure on planktonic and biofilm-exposed blastospores.

## MATERIALS AND METHODS

**Organisms.** The *C. albicans* and *C. parapsilosis* isolates used in this study were obtained by subculturing clinical specimens from the microbiology laboratory at the University Hospitals of Cleveland (29). Speciation was performed using routine germ tube tests and API20C-AUX methods. *C. albicans* strain M61 was obtained at our institution from an intravascular line culture, *C. parapsilosis* strain P/A71 was from a sputum culture, and *C. parapsilosis* strain P92 was from a blood culture. *C. albicans* strain GDH 2346 (GDH) was obtained from a patient with documented denture stomatitis (obtained from L. Julia Douglas, University of Glasgow, Glasgow, United Kingdom) and has been previously shown to produce biofilm (13, 14).

**Medium and growth conditions.** All *Candida* strains were grown in yeast nitrogen base (YNB) medium (Difco Laboratories, Detroit, Mich.) supple-

* Corresponding author. Mailing address: Center for Medical Mycology, Dept. of Dermatology, University Hospitals of Cleveland, LKSD 5028, 11100 Euclid Ave., Cleveland, OH 44106. Phone: (216) 844-8580. Fax: (216) 844-1076. E-mail: mag3@po.cwru.edu.

mented with 50 mM glucose (14). Fifty milliliters of medium (in 250-ml Erlenmeyer flasks) was inoculated with *Candida* cells from fresh Sabouraud dextrose agar plates (Difco) and incubated for 24 h at 37°C in a water bath orbital shaker at 60 rpm. Cells were harvested and washed twice with 0.15 M phosphate-buffered saline (PBS; pH 7.2; $Ca^{2+}$ and $Mg^{2+}$ free). Yeast cells were resuspended in 10 ml of PBS, counted after serial dilution using a hemocytometer, standardized to $10^7$ cells/ml, and used immediately.

**Substrate material.** Silicone elastomer (SE) sheets were obtained from Cardiovascular Instrument Corp. (Wakefield, Mass.). As per the manufacturer's instructions, the material was cleaned by washing in hand soap and water, rinsed with distilled water, and autoclaved. Flat circular disks, 1.5 cm in diameter, were obtained by cutting with a cork borer (29).

**Biofilm formation.** SE disks were placed in 12-well tissue culture plates (Becton Dickinson, Franklin Lakes, N.J.) and incubated in fetal bovine serum for 24 h at 37°C on a rocker table (14, 29). To ensure uniform biofilm formation, the SE disks were immersed in a *Candida* cell suspension. Three milliliters of standardized cell suspension, containing $10^7$ blastospores/ml, was added to the wells and the disks were incubated for 90 min at 37°C on a rocker table ("adhesion phase"). Disks were gently agitated and transferred to new plates to ensure removal of nonadherent cells. Disks were then immersed in YNB medium with 50 mM glucose and incubated for 48 h at 37°C on a rocker table ("biofilm formation phase"). For controls, disks were handled in an identical fashion except that no *Candida* cells were added. All assays were carried out in quadruplicate and on different days.

**Quantitation of biofilm.** Quantitation of *Candida* biofilms was performed as described previously (14) using both a biochemical assay, the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) reduction assay (22), and dry weight (DW) measurements. XTT (Sigma Chemical Co., St. Louis, Mo.) is reduced by mitochondrial dehydrogenase into a water-soluble formazan product that is measured spectrophotometrically. Following the biofilm formation phase, SE disks containing *C. albicans* or *C. parapsilosis* biofilms were transferred to a new 12-well tissue culture plate containing 3 ml of PBS per well. For *C. albicans* strains, 50 μl of XTT salt solution (1 mg/ml in PBS) and 4 μl of menadione solution (1 mM in acetone; Sigma) were added to each well. Due to the lower XTT metabolism of the *C. parapsilosis* P/A71 and P92 strains (29), when these isolates were assayed we used 50 μl of a 5-mg/ml XTT solution and 12 μl of menadione. Plates were incubated at 37°C for 5 h and then the medium was removed and centrifuged for 5 min at 6,000 × *g* to pellet any suspended cells or debris. XTT-formazan in the supernatant was measured at 492 nm by using a spectrophotometer (Genesys 5; Spectronic Instruments, Rochester, N.Y.). DW measures total biofilm mass, including fungal cells and extracellular matrix (ECM) (for details of this technique see references 14, 20, and 29). Briefly, biofilms were scraped off the surface of the disks by using a cell scraper (Becton Dickinson), and both disks and scrapers were rinsed with PBS to remove residual biofilm. The material collected was filtered using a 0.45-μm-pore-size Millipore filter, air dried at 37°C for 48 h, and weighed (29).

**Antifungal susceptibility.** Fluconazole (FLC) and voriconazole (VRC) were obtained from Pfizer Pharmaceuticals Group (New York, N.Y.); ravuconazole (Ravu; BMS 207-147) was from Bristol Meyers Squibb (Wallingford, Conn.); chlorhexidine (Chlor), amphotericin B (AMB), and nystatin (NYT) were from Sigma; terbinafine (TRB) was from the Novartis Research Institute (Vienna, Austria); caspofungin (Casp) was from Merck (Rahway, N.J.); micafungin (Mica; FK 463) was from Fujisawa (Deerfield, Ill.); AMB lipid complex (ABLC) was from Elan Pharmaceuticals/The Liposome Co. (Princeton, N.J.); liposomal AMB was from Fujisawa Healthcare, Inc. and Gilead Sciences (San Dimas, Calif.); and lipid complex-NYT was from Aronex Pharmaceuticals, Inc. (The Woodlands, Tex.). Drugs were solubilized in sterile saline (FLU, ABLC, Casp, Mica), sterile water (liposomal AMB), or dimethyl sulfoxide (AMB, NYT, TRB, Chlor, VRC, Ravu).

We used our published method to determine antifungal susceptibilities of biofilm-grown *Candida* isolates (14, 22, 29). Briefly, following biofilm formation, disks were gently agitated and transferred to new culture plates to remove non-biofilm-associated cells. YNB (3 ml) containing different concentrations of the antifungal agent was added to each well to produce final concentrations from 0.063 to 256 μg/ml. Biofilm activity at 48 h was determined using the XTT assay. The antifungal concentration which caused a 50% reduction in metabolic activity (50% RMA) of biofilm compared with control (incubated in the absence of drug) was then determined (22). As shown previously in planktonic cells, in this assay the 50% RMA is equivalent to the $MIC_{50}$ (minimum drug concentration at which there is 50% growth inhibition compared to control), as determined by the NCCLS M27-A method (14, 22, 35). Antifungal susceptibility of planktonic cells against the different antifungals was determined using the M27-A technique, as reported previously (13, 29, 35), and the $MIC_{50}$ was noted as the relevant endpoint (46). Resistance breakpoints were defined as follows (in micrograms per milliliter): FLC, >32; AMB, >0.5 (35, 46). For the other antifungal agents (VRC, Ravu, Chlor, NYT, TRB, Casp, Mica, ABLC, liposomal AMB, and lipid complex-NYT), clear breakpoints have not been established. As such, we defined resistance as a >5-fold increase in the $MIC_{50}$ since, conventionally, MICs within three dilutions are considered equivalent.

For CSLM of antifungal effects upon planktonic cells, *C. albicans* strain M61 was grown overnight as described above, washed with PBS, and then resuspended in 50 ml of fresh YNB medium (in 250-ml Erlenmeyer flasks) containing no drug (control), VRC (16 μg/ml), Casp (4 μg/ml), or ABLC (4 μg/ml), with a final concentration of $10^7$ cells/ml. Planktonic cells were incubated in the presence of the agents for 24 to 48 h in a water bath orbital shaker at 60 rpm. Antifungal effects on biofilms were examined in a similar fashion. At 24 to 48 h of formation, biofilms were transferred into new cell culture plates containing 3 ml of medium alone (control), VRC (16 μg/ml), Casp (4 μg/ml), or ABLC (4 μg/ml). Plates were then incubated at 37°C on a rocker for 24 to 48 h. After exposure to antifungals, planktonic cells and biofilms were prepared for CSLM imaging as below.

Subinhibitory concentration experiments were conducted using the technique of Ghannoum et al. (19) Briefly, one-fourth concentrations of the $MIC_{50}$s for individual drugs against planktonic *C. albicans* (VRC, 0.125 μg/ml; NYT, 0.5 μg/ml; Chlor, 2 μg/ml) were added to the initial culture medium of organisms ($10^7$ blastospores/ml) and 50 ml of medium. After overnight incubation, cells were washed, standardized, and used to form biofilms as described above.

**Confocal microscopy.** Biofilm staining and CSLM were performed as described previously (13, 29). FUN-1 (Molecular Probes, Eugene, Oreg.) is a fluorescent dye taken up by fungal cells; in the presence of metabolic viability it is converted from a diffuse yellow cytoplasmic stain to red, rod-like dye aggregates. Concanavalin A-alexa fluor 488 conjugate (CAAF; Molecular Probes) selectively binds to polysaccharides, including α-mannopyranosyl and α-glucopyranosyl residues, and gives a green fluorescence. Following biofilm formation and antifungal exposure, disks were removed and transferred to a new 12-well plate. Four microliters of FUN-1 (from a 10 mM stock) and 15 μl of CAAF (from a 5-mg/ml stock) were mixed into 3 ml of PBS to give final concentrations of 10 μM and 25 μg/ml, respectively. This mixture was added to wells containing biofilm disks. The plate was then incubated for 45 min at 37°C on a rocker table. Disks were removed from wells, placed in 35-mm glass-bottom microwell dishes (MatTek Corp., Ashland, Mass.), inverted, and observed using a Zeiss Axiovert 100 M confocal scanning laser microscope (using a rhodamine-fluorescein isothiocyanate protocol with excitation at 543 nm [HeNe laser] and 488 nm [argon laser], beam-splitting at 488/543 nm, and emission at 560 and 505 nm for FUN-1 [red] and CAAF [green], respectively). Lenses used included Zeiss Achroplan 20×/0.5 and C-Apochromat 40×/1.2 water immersion objectives. Images were captured and processed using Zeiss LSM510 v2.8 and Adobe Photoshop v5.5 (Adobe Systems, Inc., San Jose, Calif.) software.

For imaging of planktonic cells, a modified procedure was used. Cell suspensions were centrifuged as above and resuspended in 10 to 50 ml of medium with FUN-1 and CAAF in concentrations equivalent to those mentioned above. Several drops of stained cell suspension were placed in a cytospin apparatus and centrifuged for 8 min at 200 rpm in a Cytospin 3 centrifuge (Shandon Inc., Pittsburgh, Pa.) (15). Relevant fields on the prepared slides were then processed with Permount histological mounting medium (Fischer Scientific, Pittsburgh, Pa.) and glass coverslips were applied. Cells were imaged using CSLM as described above.

**Statistical analysis.** Each experiment was performed in quadruplicate on at least two separate days; the data shown below in the Fig. 1 and 2 are from one representative experiment. Comparative results among different isolates were normalized to those for the *C. albicans* M61 strain, which by definition was considered to have 100% activity (29). Statistical analysis, including analysis of variance post hoc analysis using the Bonferroni-Dunn calculation (4), was performed using StatView v5.0.1 software (SAS Institute, Cary, N.C.). Using the Bonferroni-Dunn calculation, $P$ values of <0.0083 were considered significant in Fig. 2, below (29).

## RESULTS

**Activities of conventional antifungals against *C. albicans* and *C. parapsilosis* biofilms.** As shown by our lab and others, mature *Candida* biofilms exhibit profound resistance to many antifungal agents (13, 21, 29). Antifungal susceptibilities of two strains each of *C. albicans* and *C. parapsilosis* were examined

TABLE 1. MICs of antifungal agents against planktonic and biofilm-associated *C. albicans* (M61 and GDH) and *C. parapsilosis* (P/A71 and P92) strains[a]

| Drug | MIC (μg/ml) in: | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Planktonically grown cells for strain: | | | | Biofilm at 48 h for strain: | | | |
| | M61 | GDH | P/A71 | P92 | M61 | GDH | P/A71 | P92 |
| AMB | 0.5 | 0.25 | 0.25 | 0.5 | 4 | 4 | 8 | 8 |
| NYT | 2 | 1 | 0.5 | 2 | 16 | 16 | 16 | 64 |
| Chlor | 8 | 8 | 8 | 8 | 32 | 8 | 16 | 64 |
| TRB | 32 | 32 | 4 | 1 | 128 | 128 | * | 128 |
| FLC | 1 | 0.25 | 8 | 1 | >256 | >256 | >256 | >256 |
| VRC | 0.5 | 8 | 0.125 | 0.03 | >256 | >256 | 128 | 256 |
| Ravu | 0.1 | 0.06 | 0.125 | 0.1 | 128 | 128 | * | 128 |
| Lip-AMB | 0.5 | 0.06 | 0.06 | 0.5 | 0.25 | 0.25 | 1 | * |
| Lip-NYT | 0.5 | 0.06 | 0.5 | 0.5 | 8 | 16 | 32 | * |
| ABLC | 0.25 | 0.06 | 0.06 | 0.25 | 0.25 | 0.25 | 0.25 | * |
| Casp | 0.125 | 0.125 | 1 | 1 | 0.25 | 0.5 | 0.125 | 4 |
| Mica | 0.001 | 0.001 | 0.25 | 0.5 | 0.25 | 0.5 | 0.125 | 2 |

[a] Results are representative of at least two separate experiments. Lip-AMB and Lip-NYT are the lipid complex formulations of AMB and NYT. For details of methods used, see text. *, unable to determine MIC.

initially. As shown in Table 1, testing of conventional agents, including FLC, AMB, NYT, Chlor, and TRB, revealed resistance by all four isolates when grown as biofilms, compared to planktonic forms. While the two new triazoles (VRC and Ravu) were effective against planktonically growing *C. albicans* and *C. parapsilosis*, they failed to inhibit the same *Candida* strains after they were grown as biofilms (Table 1). Despite repeated attempts, we were unable to determine reproducible susceptibilities for TRB and Ravu against *C. parapsilosis* strain P/A71 biofilms, perhaps due to this strain's scant, disruptable biofilms (29).

**Inhibitory activity of lipid formulations of polyene antifungals against *Candida* biofilms.** Lipid formulations of polyene antifungals have either recently come to market (liposomal AMB and ABLC) or are under development (lipid complex-NYT). Given issues of lipid complex size and the fact that standard formulations fail to demonstrate antibiofilm efficacy, lipid agents might not be expected to be effective in treating biofilm-associated *Candida*. However, as Table 1 shows, both liposomal AMB and ABLC exhibited inhibitory activities against *C. albicans* biofilms, with MICs similar to those seen for planktonic cells. *C. parapsilosis* strain P/A71 biofilms were susceptible to ABLC but had a slightly higher $MIC_{50}$ (1 rather than 0.25 μg/ml) for liposomal AMB. We were unable to determine reproducible MICs for biofilms formed by *C. parapsilosis* strain P92, perhaps due to this strain's heterogeneous biofilm formation (29). Although lipid complex-NYT showed potent activity against planktonically grown *Candida*, this antifungal failed to inhibit biofilms (Table 1). Lipid complex-NYT also thus acts as an internal control, indicating that lipid formulations per se are not the active antifungal agent (the lipid agents used in ABLC and lipid complex-NYT are the same, dimyristoyl phosphatidylcholine and -glycerol).

**Inhibitory activity of echinocandins against *Candida* biofilms.** The new class of antifungals, echinocandins, is represented by Casp and Mica in this study. The class has demonstrated effectiveness against resistant *Candida* infections (9, 17), although none of the agents are yet approved by the Food and Drug Administration for such treatment. These agents have a novel mechanism of action, blocking the production of 1,3-β-D-glucan (3), a fundamental component of the fungal cell wall. As we had shown earlier, fungal cell wall polysaccharides are components of the *Candida* biofilm ECM (13, 29). Thus, we hypothesized that the echinocandins might have activity against *Candida* biofilms. As shown in Table 1, both Casp and Mica were, in fact, active against biofilm-associated *C. albicans* (both strains). Interestingly, both echinocandins were able to inhibit *C. parapsilosis* strain PA/71 while exhibiting high MICs for strain P92.

Figure 1 shows representative curves for the inhibition of biofilm-grown *C. albicans* (strain M61) in the presence of different concentrations of FLC, AMB, Casp, and ABLC. As can be seen, there is a dramatic difference in the susceptibility plots (as measured by the XTT method) between conventional agents (FLC and AMB) and the novel drugs (Casp and ABLC). Mica and liposomal AMB produced similar curves to the latter agents; effects were similar for agents effective with strains GDH, P/A71, and P92 (data not shown).

**Effects of *C. albicans* preincubation with sub-MIC levels of antifungals on subsequent biofilm formation.** In an effort to determine if antifungal agents could affect the formation of mature biofilms, we exposed biofilm-precursor planktonic cells (*C. albicans* strain M61) to subinhibitory levels of antifungal agents. Planktonic cultures were grown in the presence of one-fourth MICs of antifungal agents and then used to form biofilms in the standard fashion. Cells grown in subinhibitory concentrations of AMB and Casp failed to form an adequate mass of planktonic cells to conduct subsequent experiments (data not shown). The results for VRC, NYT, and Chlor are

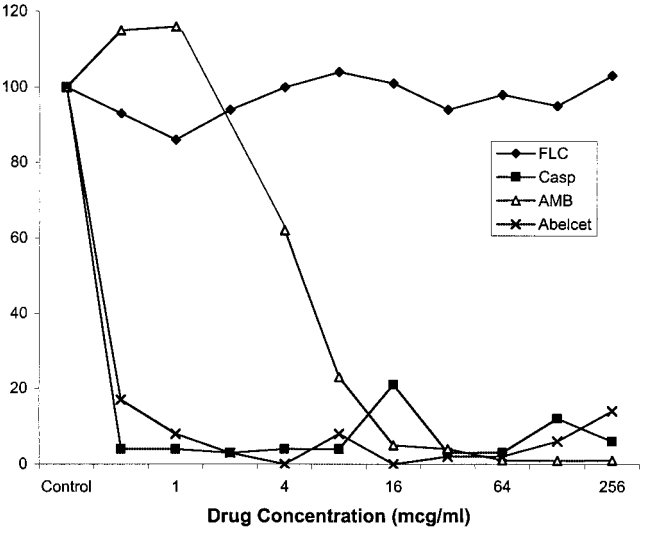


FIG. 1. Activities of different concentrations of various antifungal agents against *C. albicans* biofilms. Graph shows XTT activity of *C. albicans* strain M61 under treatment with various concentrations of antifungal agents including FLC, AMB, Casp, and ABLC. Results were normalized to control (untreated) *C. albicans* strain M61, which was taken as 100%. Each result is representative of at least two experiments. For details of methods used, see text.

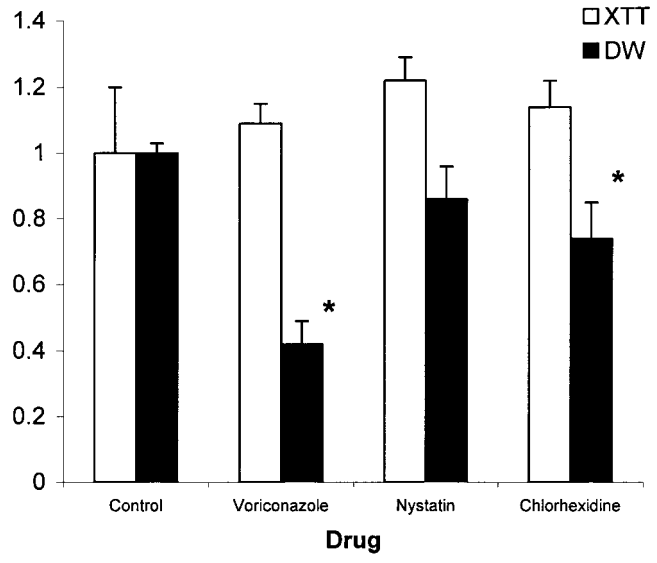


FIG. 2. Effects on biofilm production of preincubation of *C. albicans* with subinhibitory concentrations of antifungal agents. Graph shows XTT (white bars) and DW (black bars) assay results with *C. albicans* strain M61 treated with one-fourth $MIC_{50}$s of various antifungal agents. Results were normalized to control (untreated) *C. albicans* strain M61, which was taken as 1 (100%). Assays were performed in quadruplicate. Each result is representative of at least two experiments. All values are means plus standard deviations. Comparisons are significant for $P$ values of $<0.0083$. *, $P < 0.0005$ in DW assay, compared to untreated M61.

shown in Fig. 2. Results have been normalized to the control, an untreated M61 strain (29). As can be seen, pretreatment of *C. albicans* with sub-MIC levels of any of the three antifungals caused a marked decrease in the ability of cells to form a biofilm, as measured by DW ($P < 0.0005$ for comparison of VRC and Chlor with untreated control cells). This effect was maximal with VRC. At the same time, there was an increase in relative XTT activity for all three treatment groups, although the difference was not statistically significant. These findings confirm our prior results of frequent XTT-DW discordance (29).

**Confocal microscopic examination of antifungal effects on planktonic compared to biofilm-associated *C. albicans* cells.** In an effort to correlate our MIC results with *Candida* cellular and biofilm changes, we used CSLM to examine the effects of representative antifungal agents on both planktonic and biofilm-associated cells. Figure 3 shows the effects on planktonic *C. albicans* (strain M61) of Casp (Fig. 3B), ABLC (Fig. 3C), and VRC (Fig. 3D) compared with untreated control cells (Fig. 3A). Caspofungin-treated cells exhibit grossly distorted cell walls, with minimal cytoplasmic staining and no evidence of viability. ABLC-treated cells were small with diffusely yellow-staining cytoplasm, indicating lack of viability, and large vacuoles (the dark areas in the blastospores). VRC-treated cells also lacked viability. Cell wall architecture in these latter cells was distorted, but to a lesser degree than with Casp. Planktonic cells were exposed to antifungals for 24 and 48 h. Effects were the same for both groups (data not shown).

Figure 4 shows the effects of the same antifungals on *C. albicans* biofilms, specifically the basal blastospore layer (13, 29). As expected, a large number of control blastospores were still viable (Fig. 4A) (29). The most striking result was for the Casp-treated cells (Fig. 4B). Despite greatly reduced viability compared with controls (as expected from the MIC data above), there were minimal apparent distortions of cellular architecture. There were rare red FUN-1 aggregates noted. In concordance with the MIC results, ABLC-treated cells (Fig. 4C) displayed uniform nonviability, with shrunken cells and large vacuoles similar to planktonically treated cells. Also in agreement with the MIC results, VRC-treated biofilm cells (Fig. 4D) appeared minimally affected, although grossly there was an increase in the number of nonviable cells (by FUN-1 staining) and mild distortion in cell wall architecture with some apparent enlargement of blastospores compared to controls. Biofilms were grown for both 24 and 48 h, and both were exposed to antifungals for 24 and 48 h. Effects were the same for all four groups (data not shown). Due to destabilization of older (e.g., 72 to 92 h) biofilms by the CSLM dyes, we were unable to adequately visualize the entire biofilm matrix, including the hyphae that usually pervade this matrix.

## DISCUSSION

Biofilm drug resistance is a phenomenon consistently expressed across model microbial systems (6, 13, 14, 21, 29) and likely to be of great clinical relevance (16). Such resistance may explain the persistence of many infections in the face of appropriate antimicrobial therapy, ranging from difficult-to-treat *Pseudomonas* infections to *Candida* catheter-related infections and endocarditis (16, 32). As such, any evidence of activity against biofilm-associated organisms would represent an important new finding.

As predicted by earlier work (13, 14, 21, 29), our model *C. albicans* and *C. parapsilosis* biofilms are in fact uniformly resistant to a wide spectrum of conventional antifungal agents. Notably, *C. parapsilosis* biofilms appear essentially as resistant as those formed by *C. albicans*, despite being considerably less complex and forming much less ECM (29).

It is disappointing that all the biofilms were resistant to the new triazoles (VRC and Ravu), which have shown an extended spectrum of activity against many azole-resistant organisms as well as evidence of fungicidal, rather than fungistatic, activity.

Two novel classes of agents, the lipid formulation amphotericins and the echinocandins, appear to have unique activities against these *Candida* biofilms. To our knowledge, this is the first report of significant activity against *Candida* biofilms. The fact this pattern is consistent across two *C. albicans* strains and at least one *C. parapsilosis* strain strongly suggests that these results are not an artifact of chance strain selection. If these results can be extended to animal models, this could represent a breakthrough in the treatment of invasive systemic *Candida* infections. In the future, one or a combination of these drugs may allow for the retention of affected intravascular devices and other hardware or obviate the need for valve surgery within the setting of *Candida* endocarditis. Even if these drugs cannot completely fulfill this promise, there seems a likelihood that they may provide a way to treat difficult infections, or temporize when needed (i.e., allow a needed delay in device removal or surgery). Such possibilities will need to be confirmed through careful in vivo and clinical testing.

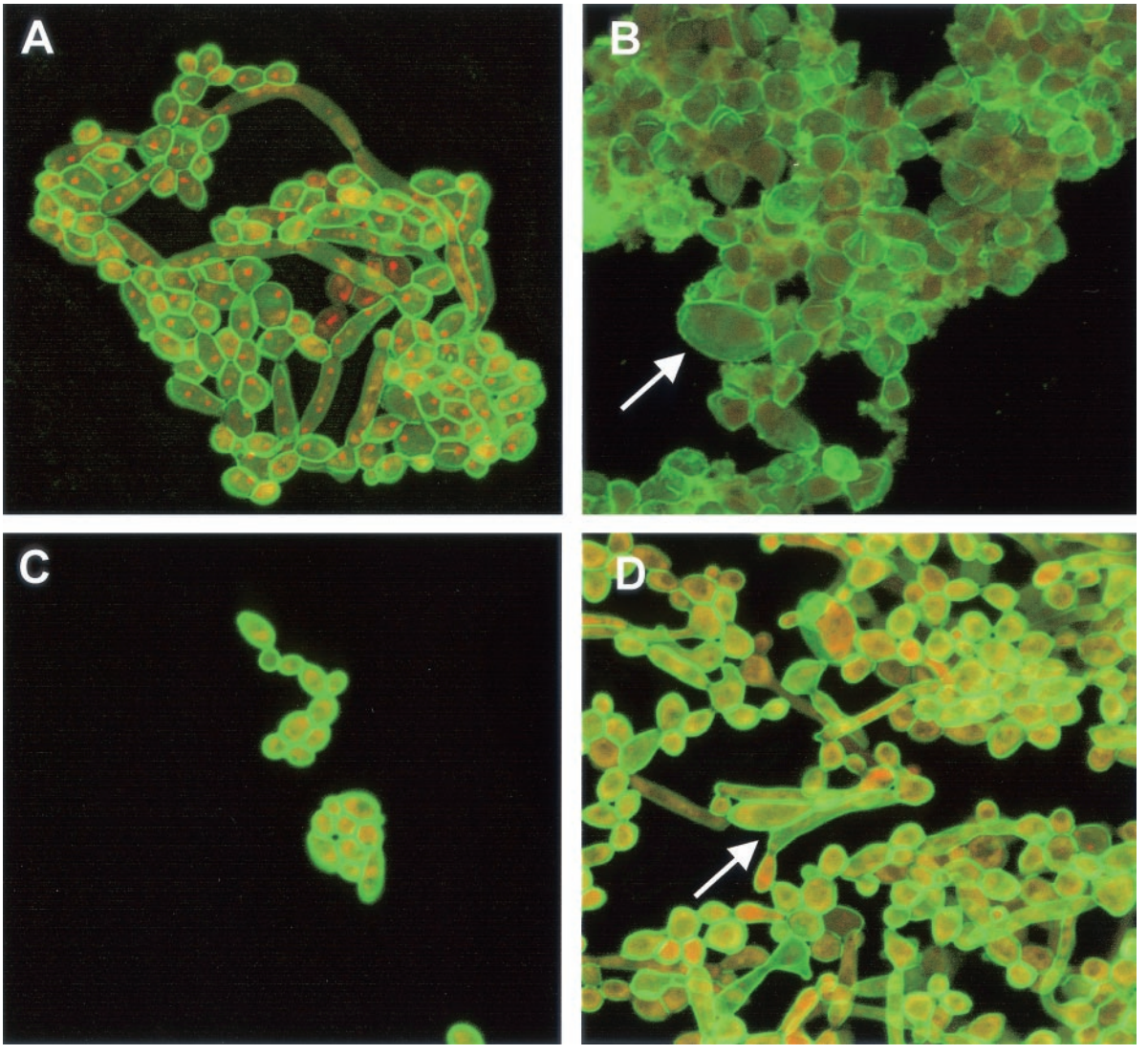


FIG. 3. CSLM of planktonic *C. albicans* cells treated with antifungal agents. Images utilize CAAF and FUN-1 staining, a 63× oil immersion objective, and 2× magnification. Green CAAF staining highlights blastospore cell walls. FUN-1 is yellow in metabolically inactive, nonviable cells; viable cells convert the stain into red-fluorescing aggregates. (A to D) Effects on planktonic *C. albicans* of treatment with Casp (B), ABLC (C), and VRC (D) compared with untreated control cells (A). Cells were exposed to antifungal agents for 24 h. Cell clumping is an artifact of the cytospin preparation. Arrows point to markedly distorted cells

Mechanisms of resistance are yet to be determined. In bacterial models, a combination of factors may be responsible, including metabolic quiescence (particularly in subpopulations, e.g., basilar cells), mitotic inhibition (32), induction of resistance elements (28, 50), reduced diffusion through and binding by ECM (7, 23, 52), and altered local microenvironments (e.g., oxygen and pH [40, 54]). We have shown previously (29) that profound metabolic quiescence does not appear to be a factor promoting antifungal resistance in *Candida* biofilms. However, more subtle metabolic down-regulation may well be occurring, in parallel to recent findings in *Pseudomonas* species (51, 58). While the relative paucity of FUN-1 conversion in biofilm-associated blastospores in Fig. 4A (compared with planktonic blastospores in Fig. 3A) may suggest this, no direct comparisons can be made due to the differing net age and processing of the specimens. Finally, it has recently been proposed that stationary-phase planktonic bacterial cells are equally as resistant, or tolerant, to antimicrobials as biofilms are (53). Ongoing work in this laboratory is examining these mechanisms in *Candida* biofilms.

The mechanisms behind the unique activities of liposomal AMB and ABLC are unknown. Given the large size of the compounds, it is somewhat surprising they penetrate the ECM to target fungal cells, although their dispersion in phospholipids may in fact facilitate passage through the charged polysaccharide ECM. Similar mechanisms may play a role in tissue penetration by these compounds (10, 55). Clearly, lipid formulation alone is not sufficient to facilitate activity against biofilms, since *Candida* biofilms were resistant to lipid complex-NYT. Moreover, the structures of ABLC (a lipid ribbon) and liposomal AMB (a true micelle) are quite different. It is unclear if the improved spectrum of ABLC versus liposomal

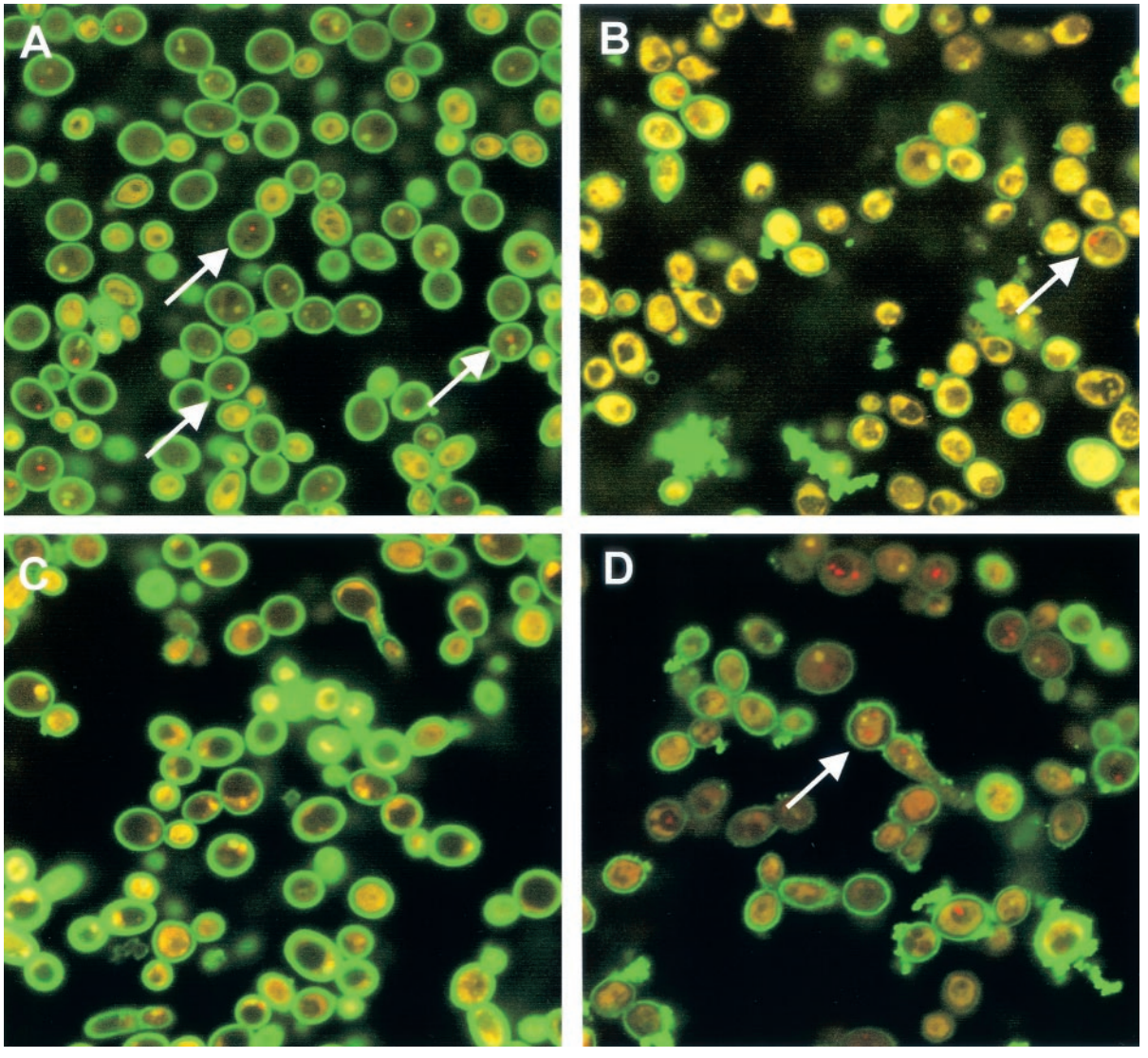


FIG. 4. CSLM of biofilm-associated *C. albicans* cells treated with antifungal agents. Images utilize CAAF and FUN-1 staining, a 63× oil immersion objective. Magnification, ×30. Each image is a Z-slice (in the *z* axis) of the basal blastospore layer. Green CAAF staining highlights blastospore cell walls. FUN-1 is yellow in metabolically inactive, nonviable cells; viable cells convert the stain into red-fluorescing aggregates. Shown are the effects on biofilm-associated *C. albicans* (specifically, the basilar blastospore layer) of Casp (B), ABLC (C), or VRC (D), compared with untreated control cells (A). Biofilm was grown for 48 h and then exposed to antifungals for an additional 48 h. Arrows point to red FUN-1 aggregates, indicating viable cells.

AMB (i.e., activity against *C. parapsilosis* biofilms), similar to results of prior in vitro studies (24, 25), is actually significant.

The echinocandins' mechanism against biofilm-associated cells is unclear, especially given the minimal cellular changes evident on CSLM images. These agents block the production of 1,3-β-D-glucan (3), a fundamental component of the fungal cell wall. Fungal cell wall polysaccharides are also important constituents of the *Candida* biofilm ECM (13, 29). If the echinocandins were exerting their antibiofilm effects primarily at the level of the fungal cell wall, one might expect that biofilm-associated blastospores would exhibit changes similar to planktonically treated cells. The lack of such changes also implies that a special ability to penetrate the ECM would not be sufficient to explain the echinocandins' efficacy. An alternate explanation may lie in a potential effect on ECM kinetics, as suggested by Lewis (31); if ECM is in a steady state, balanced between polysaccharide production and lysis, inhibition of polysaccharide production by echinocandins could lead to dissolution of the ECM and renewed vulnerability of fungal cells. Further studies to determine whether this is the case are warranted.

The reduced susceptibility to echinocandins displayed by planktonic *C. parapsilosis* strains compared with that shown by *C. albicans* (as manifested by higher, but still susceptible MICs, of the former) is not unexpected, since clinical isolates of *C. parapsilosis* are often relatively resistant to echinocandins (33). It is unclear if such apparent reduced susceptibility is clinically significant, because of variations in testing methods (42) and

the fact that in vivo models demonstrate efficacy of echinocandins against *C. parapsilosis* (1, 56). Thus, the apparent resistance to echinocandins of biofilms formed by *C. parapsilosis* strain P92 is of unknown significance.

The effects of subinhibitory concentrations of normally ineffective (against biofilms) antifungal agents on preincubating planktonic cells destined to form biofilms have several important implications. On the level of basic biofilm behavior, the results (the discrepancy between XTT and DW) confirm the validity of our earlier observations (29) that there is an inverse relative relationship between ability to produce biofilm (as measured by DW) and XTT metabolism. This is perhaps no surprise, as cells which do not form biofilm may devote more energy to routine metabolism. On a clinical level, these results may point to approaches for preventive or prophylactic treatment. Local low (hence nonsystemic) levels of antifungal agents may be sufficient to inhibit or disrupt biofilm formation, as suggested by Lewis (31). A similar principle may be applied to low-dose therapy utilizing antibiotic locks (12).

To our knowledge this is the first published report of CSLM imaging of antifungal effects of multiple drugs against *C. albicans*. Planktonic cells have largely expected changes in appearance corresponding to the mechanisms of action of the individual drugs. Casp-treated cells have severely distorted and fused cell walls, due to this drug's effects on cell wall synthesis. ABLC exposure leads to small cells with prominent vacuoles. VRC treatment produces cells with distorted morphology and deformed pseudohyphae. These effects are likely caused by the triazoles' inhibition of ergosterol synthesis and subsequent impact on the outer cell envelope (11).

Antifungal-treated, biofilm-associated *C. albicans* cells have a range of appearances. ABLC-induced changes, consisting of small nonviable blastospores with large vacuoles, are similar to those seen in planktonically treated cells and were predicted by the MIC data (showing ABLC is effective against biofilm *C. albicans*). In agreement with the MIC-measured effectiveness of echinocandins, Casp-treated blastospores are nearly uniformly nonviable. However, the Casp-treated cells exhibit nowhere near the degree of cellular distortion seen in planktonically treated cells. This may suggest a novel mechanism of echinocandin action. Additionally, there is clearly a small residual population of viable cells, in marked contrast to the MIC results. Whether these cells represent a population of "persisters" (31, 53) remains to be determined. Finally, while VRC-treated cells are minimally affected (as predicted by the MIC data), they are not normal appearing. This suggests that despite high MICs for the triazoles against biofilm-associated cells, the drugs may be exerting some subtle effects. In summary, the CSLM results point to this technique as a valuable adjunctive test for examining the effects of antifungal agents on biofilms, since it elucidates information not gleaned from MIC data.

A better understanding of how antifungals affect *Candida* biofilms may be obtained by determining the fungicidal (minimal fungicidal concentration, MFC) effects of these agents. If the observed MICs do not represent actual fungicidal activity, then in a clinical setting cessation of antifungal therapy could lead to the reappearance of organisms. This possibility may be suggested by the CSLM results, particularly for the echinocandins. However, apparent viable cells may represent a trailing phenomenon, which may not be of clinical relevance (5, 44). While MFC determinations may be more relevant to clinical outcomes, such methods have yet to be standardized (46). Our lab is currently involved in ongoing work to standardize a biofilm MFC assay. Another potential limitation exists with regard to the validity of MIC testing itself, especially in relation to certain drugs, as in vitro results do not always correlate with treatment outcomes (35, 45, 46). The M27-A method has a limited ability to detect AMB resistance (43); more significantly, the clinical relevance of susceptibility testing of newer drugs, especially the echinocandins, is unknown (30, 46).

In conclusion, two new classes of antifungal agents, the AMB lipid formulations and the echinocandins, exhibit novel activity against *Candida* biofilms. Additionally, exposure of planktonic *Candida* to subinhibitory concentrations of antifungal agents inhibits subsequent biofilm formation. Both of these findings now need to be validated using in vivo models. Finally, CSLM appears to be a valuable new tool in examining the effects of antifungal agents upon both planktonic yeast cells and fungal biofilms.

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